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14. ABSTRACT

Neurofibromatosis-I (NF-1) is an autosomal dominant genetic disorder commonly associated with cognitive impairments, including low IQ, learning disabilities, behavioral difficulties, executive dysfunction and language-based deficits. Despite the growing recognition of the importance of SK channels in cognition, there has been no previous assessment of CNS SK channels in NF-1. We were the first to investigate potential mechanisms of cognitive impairment in NF-1 at the molecular level involving potassium channels, and demonstrated a possible mechanism for the learning deficits seen in $NF1^{+/-}$ mice and a possible drug therapy for rescuing these deficits. We anticipate that better understanding of this phenomenon will potentially lead to improved treatment of patients who are cognitively impaired due to NF-1.

15. SUBJECT TERMS
Neurofibromatosis-1, potassium channel, SK channel, cognitive impairement

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INTRODUCTION

Neurofibromatosis-I (NF-1) is an autosomal dominant genetic disorder commonly associated with cognitive impairments, including low IQ, learning disabilities, behavioral difficulties, executive dysfunction and language-based deficits. In mice, NF-$1^{+/-}$ mutation also affects learning and memory. As in humans, learning and memory deficits are restricted to specific types of learning, they are not fully penetrant, they can be compensated for with extended training, and they do not involve deficits in simple associative learning.[1] Small conductance Ca^{2+}-activated potassium (SK) channels are found in neurons and astrocytes of the CNS. These channels are selective for potassium, and open when intracellular Ca^{2+} exceeds 300 nM. Three isoforms of SK channels (SK1, SK2, SK3) have been cloned from brain. Proper function of SK channels has been implicated in the control of memory and cognition,[2] and SK-channel blockers such as apamine, dequalinium and UCL 1684, are considered to be cognitive enhancers.[3] Abnormal activation of astrocytes is common in the brain of NF-1 patients,[1] but an ionic channel basis for this has not been identified. Potassium channel currents are abnormal in peripheral nerve tumor Schwann cells from NF-1 patients and from NF-1 models.[4] However, to date no study has examined ion channels, including SK channels, in non-neoplastic astrocytes or neurons in NF-1.

We hypothesize that cognitive impairment in NF-1 is related to constitutive up-regulation of SK channel expression in CNS cells, including neurons and astrocytes. We evaluated this hypothesis by: (i) comparing regional expression of SK channels in brain, (ii) comparing SK channel function in astrocytes, and (iii) comparing the cognitive effect of SK channel inhibition in wild type (WT) vs. NF-$1^{+/-}$ mice.

BODY

I will describe the research accomplishments associated with each task outlines in the approved Statement of Work in my proposal.

Task 1: Perform qualitative and quantitative regional brain mapping of SK channel isoform distribution in WT vs. NF-$1^{+/-}$ mice.

We first looked at SK channel protein expression in different region of brain. Using immunohistochemical technique, we found that SK1 expression was upregulated in NF-$1^{+/-}$ mice when compared to the WT brains. The upregulation of SK1 was more prominent in the hippocampus and olfactory tract. As previously reported before[5], we also found that NF-$1^{+/-}$ mice had higher GFAP, an astrocyte marker, expression in the hippocampus and the neocortex (Figure 1). However, we didn't detect another two isoforms of SK channel, SK2 and SK3, expression in either NF-$1^{+/-}$ or WT brains. Using *in situ* hybridization techniques, we also mapped three isoforms of SK channel mRNA distribution in these mice, and found that only SK1 mRNA was present in a higher amount fashion in the similar distribution as shown in immunohistochemistry.

To correlate with these findings, we did Western blots to look at SK1 and GFAP levels in the brain. Western blot analyses indicated a significantly higher amount of SK1 expression when normalized with β-actin in NF-$1^{+/-}$ mouse brains (M= 0.44, SD= 0.104) when compared to WT brains (M= 0.21, SD= 0.051), $t(5)$= -3.36, p= 0.028. However, GFAP expression in NF-$1^{+/-}$ mouse brains (M= 0.60, SD= 0.033) was not different from WT brains (M= 0.56, SD= 0.10), $t(5)$= -0.63, p= 0.56 (Figure 2). The increase in SK1 protein was corroborated by measurements of mRNA for SK1, which increased 3-4-fold in the areas of hippocampus and olfactory tract.

Task 2. Assess functional SK channels in regional astrocytes isolated from regions of interest identified in Task 1.

We implanted small pieces (1x1x1 mm) of gelatin sponge into brains of WT and NF-$1^{+/-}$ mice. After 1 week *in situ*, we did study to look at the brain responses to these implants, and found out that NF-$1^{+/-}$ mouse brain had stronger reaction to gelatin sponge, i.e., there were more astrocytes migrating into the implants. As further studied, these migrating cells were GFAP positive, indicating that NF-$1^{+/-}$ mice had a stronger astrocyte reaction to foreign body compared to WT mice (Figure 3).

We enzymatically isolated these astrocytes and grew them for patch clamp study. Using standard patch clamp techniques, we found that astrocytes isolated from NF-$1^{+/-}$ mice had larger membrane current to WT mice (Figure 4). These outward current could be abolished by 0.1 μM of apamin, a specific SK1 channel inhibitor, further proved that NF-$1^{+/-}$ brain had higher expression of SK1 channels.

T**ask 3**. Assess the ability of the specific SK-channel inhibitor, UCL 1684, to improve cognitive function in WT vs. NF-$1^{+/-}$ mice.

Using a "Visible water maze" test, which is abnormal in NF-$1^{+/-}$ mice,[4] we assessed cognitive function in WT and NF-$1^{+/-}$ mice. UCL 1684 significantly increased swimming speed in both genotypes, F (2, 78) = 3.77, p = .027. We therefore analyzed the cumulative distance of the swim path from the target platform. This measure is independent of the speed of which the animals completed the task. The four daily spatial trials were averaged to create one measure per day. A three-way mixed ANOVA (Day x Genotype x Drug) indicated a significant decrease in cumulative distance over training Days, F (5, 395) = 78.22, $p < 0.001$ (Figure 5). Modified Bonferroni t-tests indicated significant differences between all daily averages (all p's < 0.003) with the exception of day 4 (M =15.65, SD = 7.26) and day 5 (M =14.72, SD =8.43), t (84) = 1.07, p = 0.29. There was a near significant effect of Genotype F (1,79) = 3.12, p = 0.08, a near significant interaction between Day and Drug, F (10 , 395) = 1.65, p = 0.09, and a near significant three-way interaction between Day, Drug, and Genotype, F (10, 395) = 1.65, p = 0.08. Planned comparisons indicated that NF-$1^{+/-}$ animals given saline performed significantly worse (had a higher cumulative distance) than WT animals given saline on day 3 (t (37) = 2.93, p = 0.006), day 4 (t (37) = 2.99, p = 0.005), and day 5 (t (37) =2.77, p = 0.009; Figure 5). NF-$1^{+/-}$ animals given 0.2 mg/kg (M = 17.57, SD = 6.95) and NF-$1^{+/-}$ animals given 0.4 mg/kg of UCL 1684 (M = 16.38, SD =9.2) had a significantly

lower cumulative distance on day 3 in comparison to NF-$1^{+/-}$ animals given saline (M= 24.77, SD = 6.67), t (27) = 2.85, p = 0.008; t (31) = 3.03, p = 0.005. NF-$1^{+/-}$ animals given either 0.2 mg/kg or 0.4 mg/kg were not different from any WT animals (all p's > 0.05). Thus, UCL 1684 had no effect on cumulative distance in WT animals (all p's > 0.05).

Probe tests also indicated an improved performance in NF-$1^{+/-}$ animals following UCL 1684 treatment. A three-way mixed ANOVA (Probe Number x Genotype x Drug) was used to examine dwell time in the target quadrant during the probe trials. There was a significant increase in dwell time over the three probe tests, F (2,158) = 44.18, p < 0.001 (Figure 6). Modified Bonferroni t-tests indicated a significant increase in dwell time between Probe 1 and Probe 2, t (85) = 8.25, p < 0.001, and a significant increase between Probe 1 and Probe 3, t (84) = 8.61, p < 0.001. There was a significant interaction between the Probe Number and Drug, F (4,158) = 2.87, p = 0.025. Post-hoc comparisons indicated NF-$1^{+/-}$ animals given saline had a significantly lower dwell time than WT animals given saline on Probe 2, t (37) = 2.99, p = 0.005. NF-$1^{+/-}$ mice given 0.2 mg/kg of UCL 1684 had a significantly higher dwell time than NF-$1^{+/-}$ animals given saline on Probe 2, t (31) = 3.19, p = 0.003. Wild-type animals given 0.4 mg/kg of UCL 1684 had a significantly lower dwell time than WT animals given saline on Probe 2, t (30) = 3.36, p = 0.002.

In summary, administration of 0.2mg/kg and 0.4mg/kg dose of UCL 1684 either through i.p injection or micro-osmotic pump to NF-$1^{+/-}$ mice significantly improved performance on the water maze task in comparison to saline treated NF-$1^{+/-}$ mice on the third day of training and on the corresponding probe test.

Task 4. Perform data analysis, write a report, and submit the report for publication.

We are writing a manuscript targeting at Proceedings of the National Academy of Sciences.

KEY RESEARCH ACCOMPLISHMENTS

- We found that SK1 channel was upregulated in NF-$1^{+/-}$ mouse brain, especially in the hippocampus and olfactory tract regions.
- NF-$1^{+/-}$ brain had a stronger astrocyte reaction to gelatin implant compared to WT brain.
- Astrocytes isolated from NF-$1^{+/-}$ mouse brain had different outward current profile to WT astrocytes.
- The increased outward current in NF-$1^{+/-}$ astrocytes can be blocked by apamin, a specific SK1 channel blocker.
- Administrating another SK1 channel blocker,UCL 1684, to NF-$1^{+/-}$ mice either through i.p. injection or micro-osmotic pump delivery, can significantly improved performance on the water maze task in comparison to saline treated NF-$1^{+/-}$ mice on the third day of training and on the corresponding probe test.

REPORTABLE OUTCOMES

Part of this work was presented at 2005 Society of Neuroscience Annual Meeting in Washington , DC.

M. Chen, A. Kallarackal, Z. Li, N. Dugger, P. Hoffman, J. Simard. Small-conductance calcium-activated potassium (SK) channels in neurofibromatosis-1 mouse brains. Program No. 376.14. *2005 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, 2005. Online.

CONCLUDIONS

Despite the growing recognition of the importance of SK channels in cognition, there has been no previous assessment of CNS SK channels in NF-1. We were the first to investigate potential mechanisms of cognitive impairment in NF-1 at the molecular level involving potassium channels, and demonstrated a possible mechanism for the learning deficits seen in $NF1^{+/-}$ mice and a possible drug therapy for rescuing these deficits. We anticipate that better understanding of this phenomenon will potentially lead to improved treatment of patients who are cognitively impaired due to NF-1.

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APPENDICES

Abstract presented at 2005 Annual Society of Neuroscience Meeting.

Small Conductance Calcium Activated Potassium (SK) Channels in Neurofibromatosis-1 Mouse Brains

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St. Mary College[2], St. Mary, MD, USA
Maryland VA Medical Center[3], Baltimore, MD, USA

Introduction & Objective: Neurofibromatosis-1 (NF-1) is a common AD disorder, and is associated with cognitive impairments. In a murine NF-1 model, brain tissue of NF-$1^{+/-}$ shows upregulated GFAP expression, a marker of astrocyte activation. Astrocytes derived from NF-$1^{+/-}$ mice display enhanced proliferation and motility in vitro. In these mice, NF-$1^{+/-}$ mutation also affects learning and memory. K channel currents are abnormal in neoplastic Schwann cells from NF-1 patients. However, no study has examined ion channels in non-neoplastic astrocytes in NF-1.

Methods: We examined NF-$1^{+/-}$ mice in a CNS injury model in which a gelatin sponge is implanted in the parietal cortex to retrieve reactive astrocytes. Patch clamping was used to study the electrophysiology of isolated astrocytes. We also used immuno-labeling and Western blotting to examine SK channel distribution/expression in these mice.

Results: Cells in gelatin implants distinguished NF-$1^{+/-}$ astrocytes from NF-$1^{+/+}$ controls, in that NF-$1^{+/-}$ astrocytes express high levels of K channel activity. We also showed that high level of K current in NF-$1^{+/-}$ astrocytes could be blocked by apamine, a specific SK channel blocker. We further investigated SK channel expression in CNS, and found a significant up-regulation of SK1 expression in NF-$1^{+/-}$ brain. The highest areas of up-regulation were hippocampus and olfactory tract, both of which are areas associated with learning.

Conclusions: Our results suggest that functioning of the hippocampus and olfactory tract is being altered by the abnormal influx of SK1 current. Proper function of SK channels has been implicated in the control of memory and cognition, and SK channel blockers such as apamine are considered to be cognitive enhancers. We expect this study will shed new light on the learning deficiency in NF-1, and will point to new therapeutic targets to cognitive dysfunction in NF-1.

SUPPORTING DATA

FIGURE LEGEND

Figure 1. SK1 and GFAP immunohistochemistry analysis at 0.5x magnification of whole brain slices of $NF1^{+/-}$ and WT samples. SK1 immunohistochemistry analysis at 40x magnification hippocampus in whole brain slices of representative WT and $NF1^{+/-}$ samples.

Figure 2. SK1, GFAP and β-actin western blot analysis on same membrane of $NF1^{+/-}$ and WT whole mouse brain samples. Each band represents an individual brain. Bar graph includes mean band densities and standard deviation, calculated after normalizing with β-actin bands on same membrane. $NF1^{+/-}$ brains showed a significant increase in SK1 channels ($p = 0.028$).

Figure 3. Representative sections of gelatin implantation in $NF1^{+/-}$ and WT mice, showed a stronger astrocyte reaction in $NF1^{+/-}$ brain.

Figure 4. Electrophysiology recording showed increased outward currents in $NF1^{+/-}$ astrocytes.

Figure 5. a. Mean (± SE) cumulative distance from the target platform over six days of training. $NF1^{+/-}$ mice given either 0.2 mg/kg ($p = 0.008$) or 0.4 mg/kg ($p = 0.005$) performed significantly better than $NF1^{+/-}$ mice given saline on Day 3. b. Representative samples of the swim path on day 3 for each treatment group are shown.

Figure 6. Mean (± SE) dwell time in the target quadrant over three probe tests. On Probe 2, $NF1^{+/-}$ animals given 0.2 mg/kg of apamin spent significantly more time in the target quadrant than $NF1^{+/-}$ animals given saline ($p = 0.003$).

Figure 1

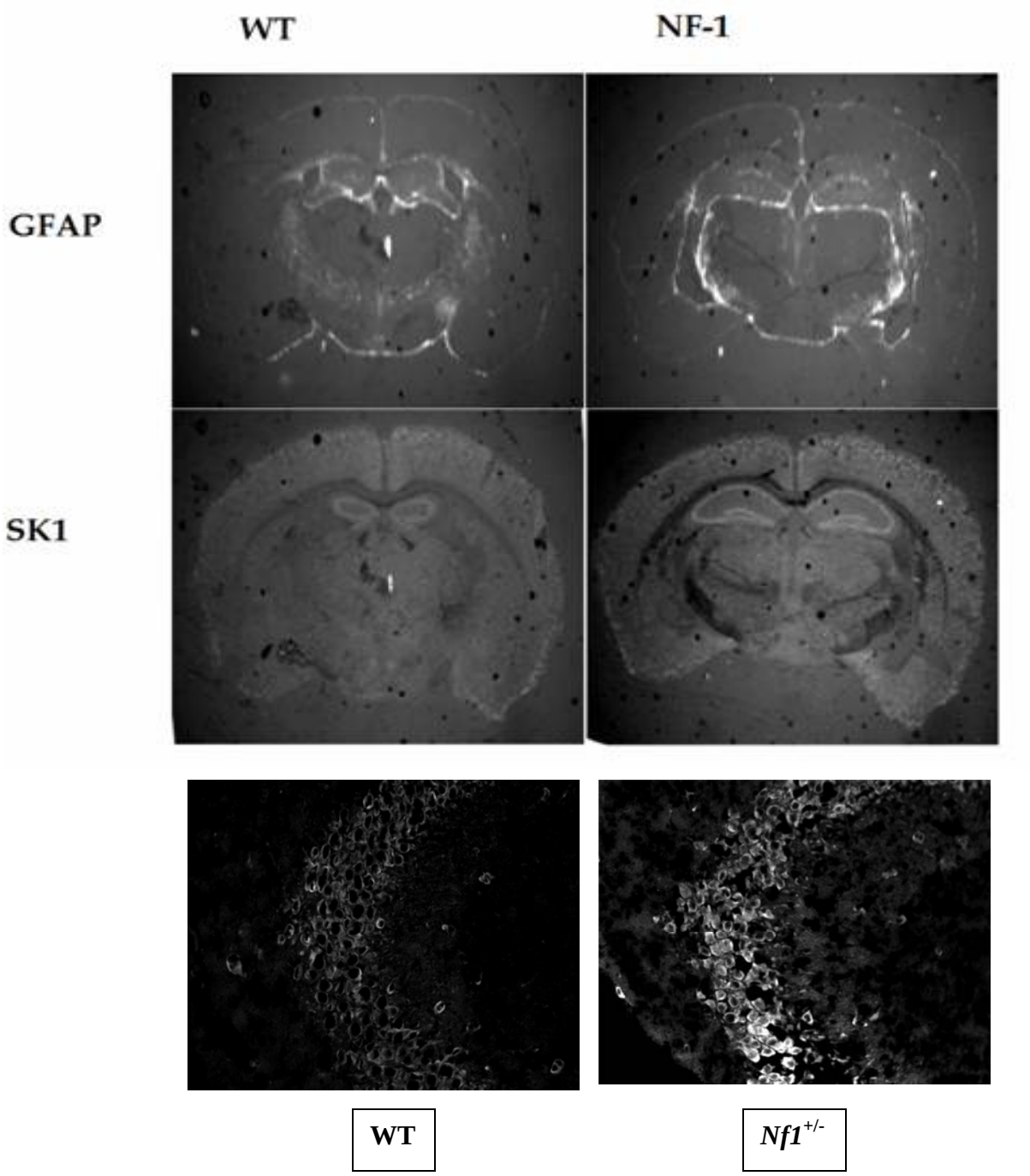
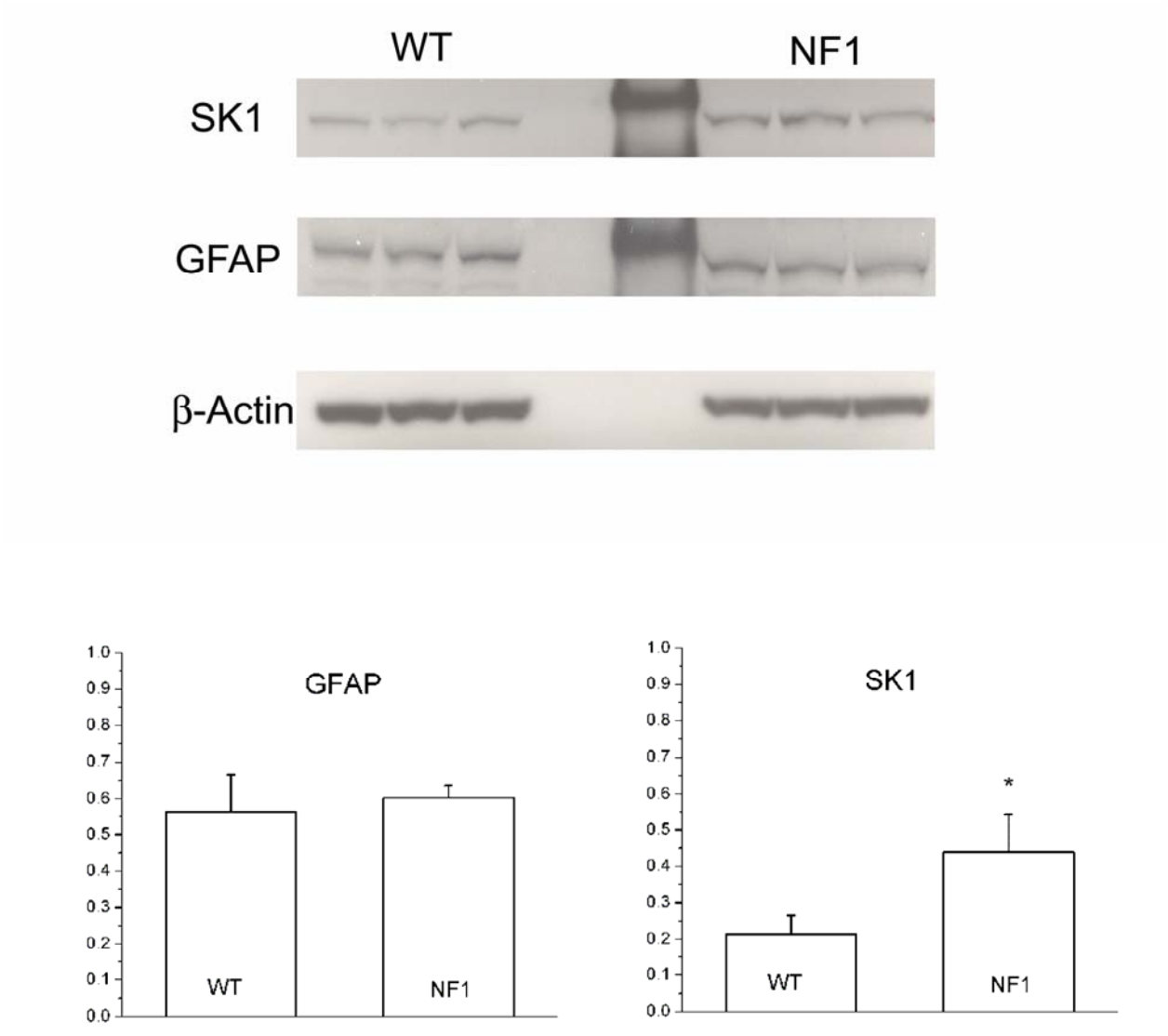


Figure 2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NF1
SK1
GFAP
β-Actin
GFAP
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0.8
0.7
0.6
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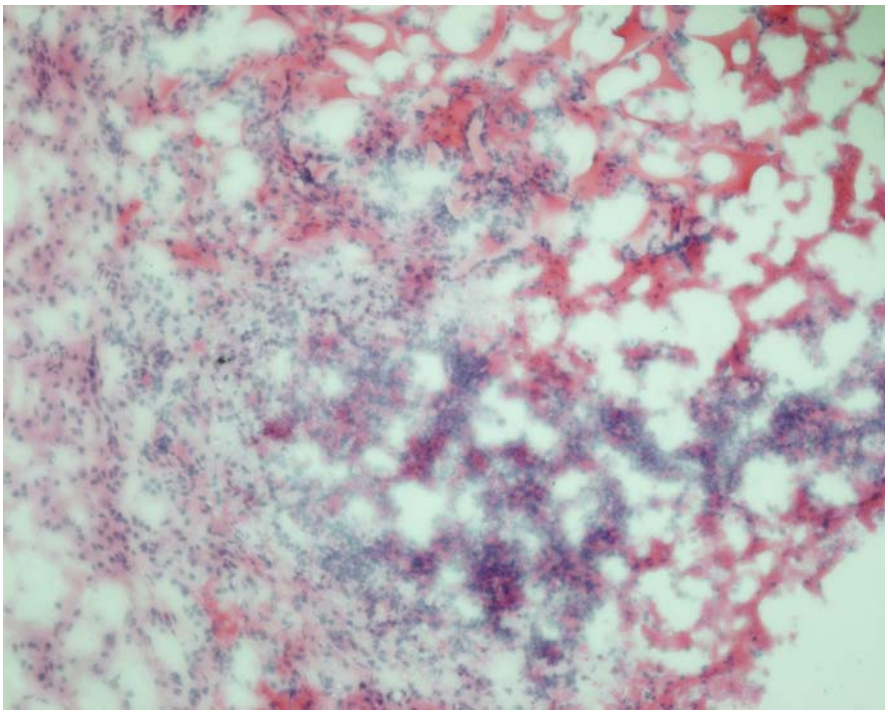
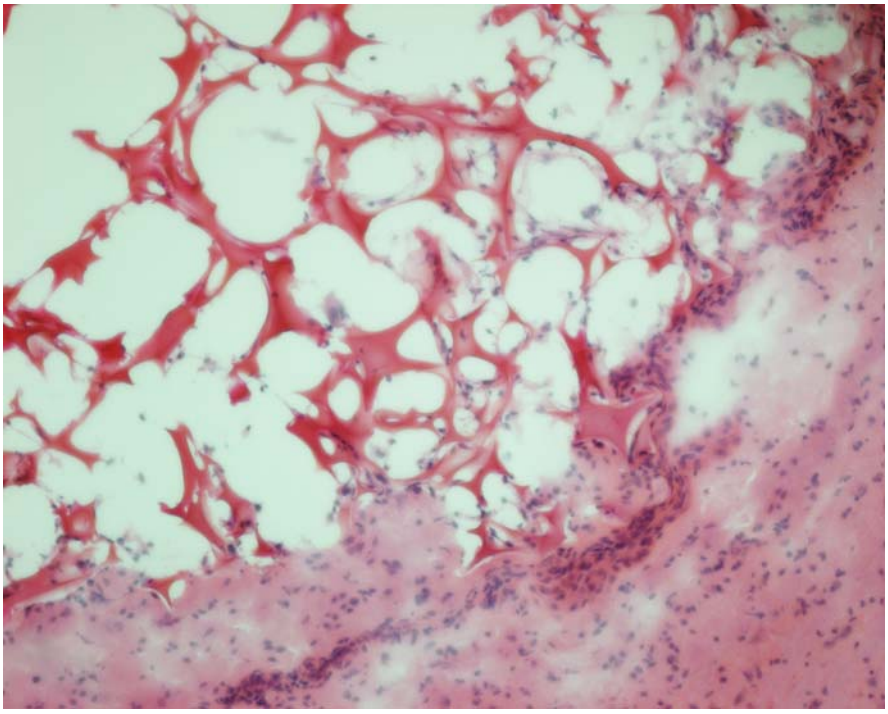
0.3
0.2
0.1
0.0
WT
NF1
SK1
*
WT
NF1

Figure 3



NF1$^{+/-}$



WT

Figure 4

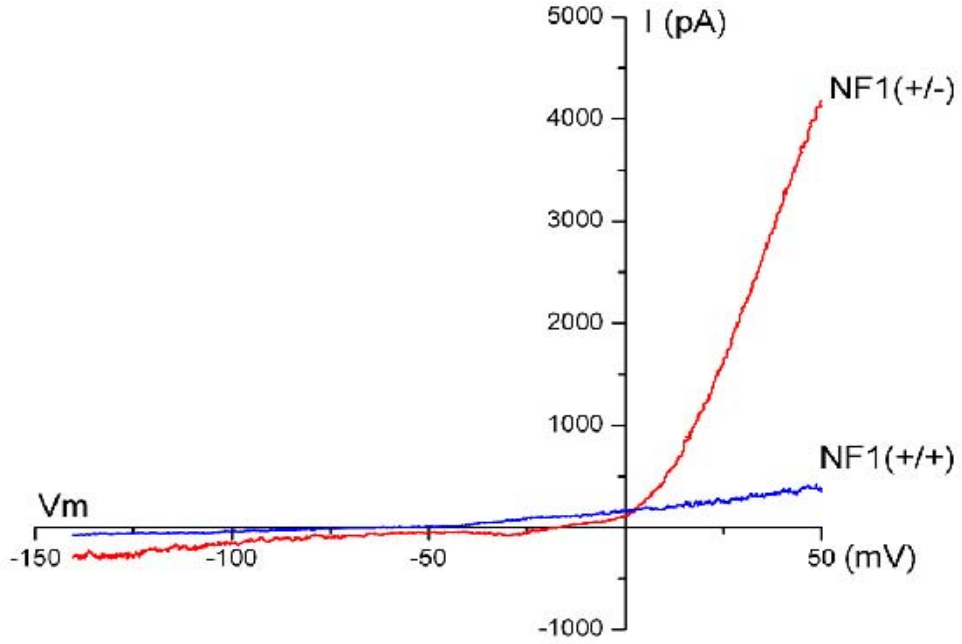
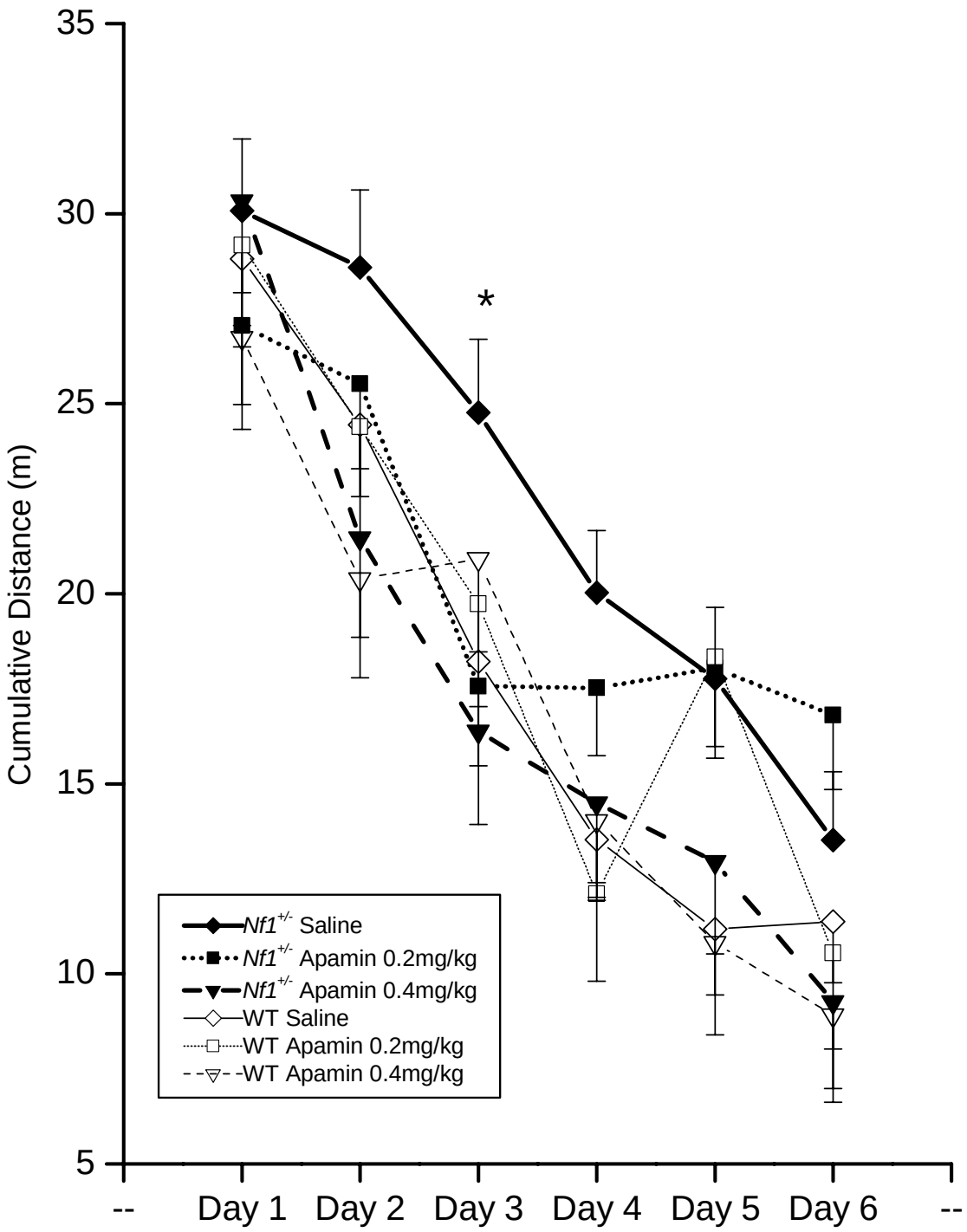


Figure 5a


35
30
25
20
15
10
5
Cumulative Distance (m)
*
Nf1+/- Saline
Nf1+/- Apamin 0.2mg/kg
Nf1+/- Apamin 0.4mg/kg
WT Saline
WT Apamin 0.2mg/kg
WT Apamin 0.4mg/kg
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Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
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Figure 5b

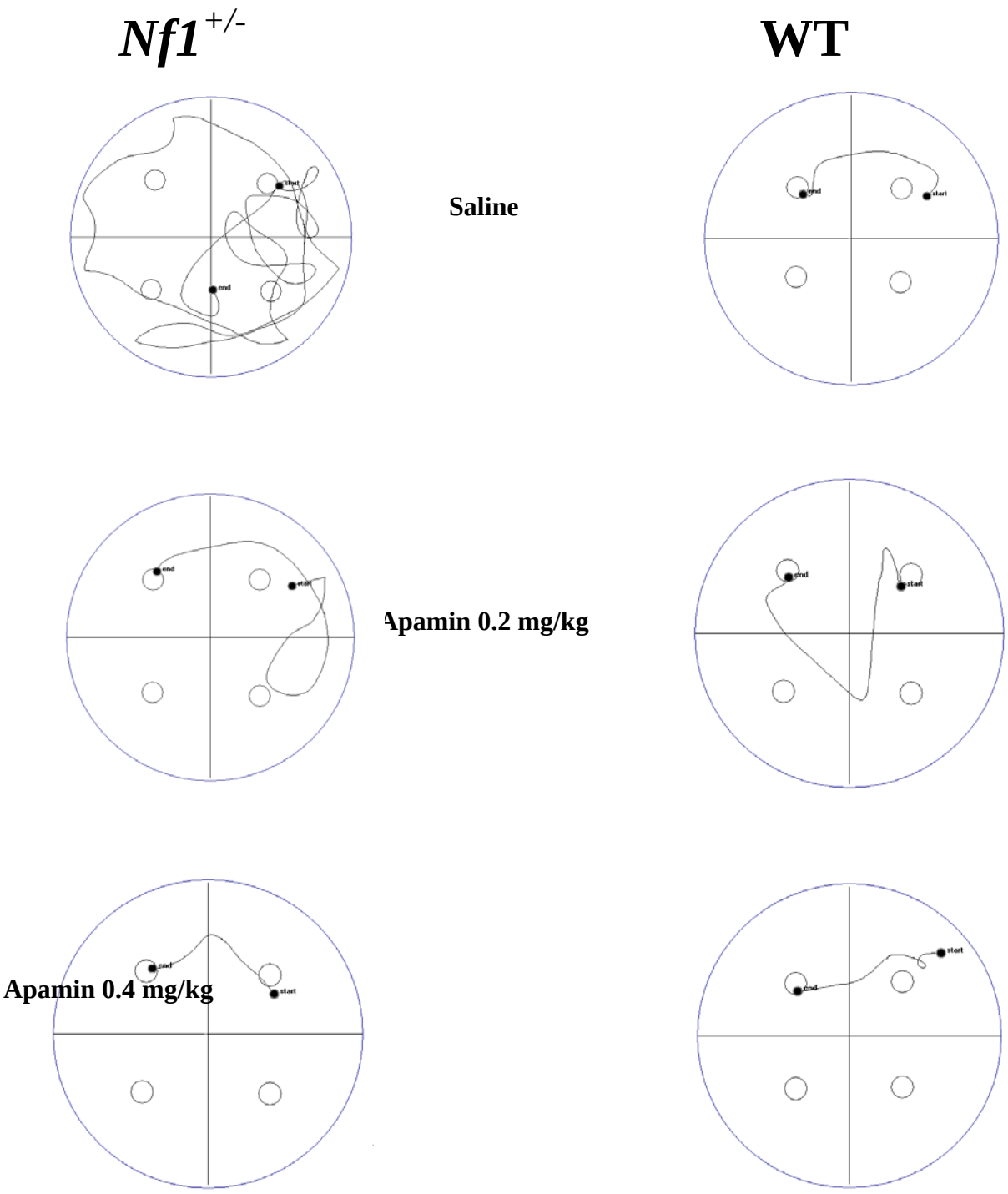
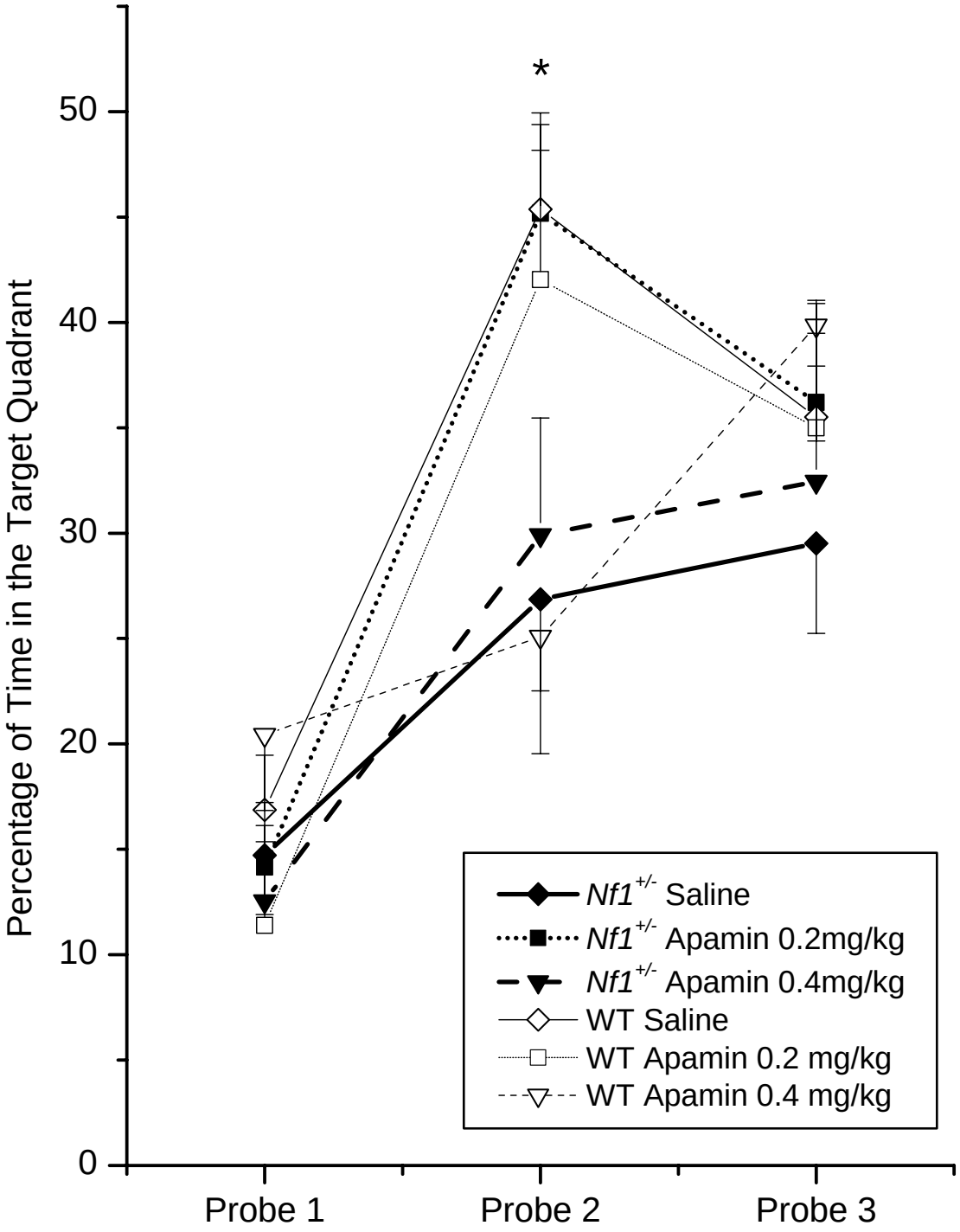


Figure 6


Percentage of Time in the Target Quadrant
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10
20
30
40
50
*
Probe 1
Probe 2
Probe 3
Nf1+/- Saline
Nf1+/- Apamin 0.2mg/kg
Nf1+/- Apamin 0.4mg/kg
WT Saline
WT Apamin 0.2 mg/kg
WT Apamin 0.4 mg/kg